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Acidification and Stress Physiology in a Plethodontid Salamander, Desmognathus ochrophaeus

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ACIDIFICATION AND STRESS PHYSIOLOGY IN A PLETHODONTID SALAMANDER, *DESMOGNATHUS OCHROPHAEUS*

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By

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ABSTRACT

ACIDIFICATION AND STRESS PHYSIOLOGY IN A PLETHODONTID SALAMANDER, *DESMOGNATHUS OCHROPHAEUS*

By

Lauren F. Ricciardella

June 2008

Thesis Supervised by Dr. Sarah Woodley

In western Pennsylvania, chronically and episodically acidified stream habitats are common. The mountain dusky salamander, *Desmognathus ochrophaeus*, is an abundant species that is associated with acidified streams. The focus of this research was to (1) examine the effects of acidified habitats on the stress hormone, corticosterone, and (2) examine the effects of corticosterone on behavior. In an acidified stream site, males had a blunted corticosterone stress response after capture and handling compared to an acid neutral site. There was a trend for elevated corticosterone to inhibit several aspects of male mating behavior, including insemination of females. These data suggest that acidification may alter normal stress physiology and thereby influence the expression of mating behavior. Ultimately, this data would help to understand how environmental degradation (specifically degradation associated with acidification) affects behaviors in free living animals via the effects on stress physiology.

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LIST OF ABBREVIATIONS

ACTH Adrenocorticotropic Hormone

ANC Acid-Neutralizing Capacity

BHT 2,6-di-tert-butyl-p-cresol

CORT Corticosterone

CRH Corticotrophin Releasing Hormone

CV Coefficient of Variation

FSH Follicle Stimulating Hormone

GnRH Gonadotropin Releasing Hormone

LH Luteinizing Hormone

MEHQ 4-methoxyphenol

PBS Phosphate Buffered Saline

PEGDA Poly (ethylene glycol)diacrylate

QT Pentaerythritol tetrakis 3-mercaptopropionate

RIA Radioimmunoassy

Introduction

1. Significance and Aims

In western Pennsylvania, chronically and episodically acidified streams are common. The mountain dusky salamander, *Desmognathus ochrophaeus*, is an abundant local species that is associated with acidified streams. However, little is known about how acidification affects their endocrine physiology. The focus of this research is to more fully understand the effects of acidification on the physiological stress response of stream-side salamanders by (1) studying their stress response in the field in an episodically acidified site and an acid neutral site, and (2) studying their behavioral responses to chronically elevated corticosterone (a stress hormone) in the laboratory. The behaviors studied were mating, feeding, and activity level in the presence or absence of chemosensory cues. Ultimately, I would like to understand how environmental degradation (specifically degradation associated with acidification) affects behaviors in free living animals via the effects on stress physiology.

2. Environmental Acidification

Episodic acidification is defined as short-term decreases in the acid-neutralizing capacity (ANC) of surface waters during hydrological events such as heavy precipitation or snowmelt. This can result in short-term loss of ANC causing, for example, increased concentrations of hydrogen ions and metals such as aluminum (Vertucci and Corn, 1996). Episode severity varies among regions and also varies among streams and lakes within a

region. Episodic acidification can cause ANC values of less than 0 and pH values of less than 5.0 (Wigington, et al., 1996). During normal base flow stream conditions, alkaline water is derived from the lower part of the mineral soil and groundwater storage zones. During hydrological events, however, water is routed through upper soil layers which may be more acidic due to natural processes or acidic deposition (Wigington, et al., 1996).

Acid precipitation can be categorized as naturally occurring or anthropogenic. Naturally occurring sources include volcanoes and natural processes of wetlands and oceans. Anthropogenic sources include emissions from fossil fuel burned in power plants and automobiles (Vatnick, et al., 2006). Specifically, precipitation can provide direct inputs of acidic water to surface waters, introduce SO_4^{2-}, NO_3^-, NH_4^+, and H^+ to the water from the upper layers of watershed soils during dry periods, and lower the chronic ANC of the system thereby lowering the minimum ANC values attained during episodes (Wigington, et al., 1996).

Many northeastern states, including Pennsylvania, suffer from acid precipitation, and while acid precipitation has been reduced because of the Clean Air Act of 1963, many watersheds are still plagued with acidification possibly due to poor natural buffering capacity in the soil and water (Vatnick, et al., 2006) and soil acidification from maturing deciduous forests (Wyman and Hawksley-Lescault, 1987).

Amphibians are highly susceptible to acidification because of their close association with the water and surrounding soil. Acidification impairs osmoregulation and sodium balance which can lead to toxic and lethal effects. Sodium balance in the red-spotted newt (*Notophthalmus viridescens*) was affected by changes in pH. In an

aquatic setting of pH 5.0, sodium balance was maintained; however, in a pH of 3.0, sodium was lost over a 48 hour period (Frisbie and Wyman, 1992). In addition, long term exposure (14 days) of low pH waters to Jefferson's salamander (*Ambystoma jeffersonianum*) caused a decrease in whole body water and Na^+ concentrations compared to exposure to higher pH waters (Horne and Dunson, 1994).

Studies on the red-backed salamander (*Plethodon cinereus*) by Wyman and Hawksley-Lescault (1987) found that the salamanders avoided soils with a pH that falls to 3.7 and this is particularly true for juvenile salamanders. Hypotheses for this occurrence are that juvenile salamanders die at low pH, adults move away from low pH soil to lay eggs in high pH soil, little to no food could be found in low pH soil, and/or the size of the juvenile salamanders are smaller in low pH soil so they could fall easier to predation.

Acidification can have additional physiological effects on amphibians. Kiesecker (1996) reported that the ability of the tiger salamander larvae (*Ambystoma tigrinum*) to capture tadpole prey was reduced at lower pH levels. Regardless of pH, attempts were made to eat tadpoles at all pH levels; however, predation attempts were unsuccessful at lower pH levels. When placed on substrate with pH 3.5 to 5.0, the black-bellied salamander (*Desmognathus quadramaculatus*) and seal salamander (*Desmognathus monticola*) showed an inhibition of feeding when compared to animals placed on a substrate of pH 7.2, indicating that feeding behavior is acid sensitive (Roudebush, 1988).

Acidification can also influence morality rate by affecting the inflammatory response. By exposing northern leopard frogs (*Rana pipiens*) to acidic environments, Vatnick, et al. (2006) found a decrease in the influx of leukocytes to the peritoneal cavity,

weakening the inflammatory response. Also, there was a decrease in the phagocytic function of the leukocytes as well as a decrease in the number of leukocytes at the inflammatory site. They concluded that environmental stress may be the initiating factor leading to immunosuppression of the innate and adaptive immune response.

3. Vertebrate Stress Response

The stress response consists of a suite of physiological and behavioral adaptations made by the body to reestablish homeostasis after a challenge (Nelson, 2000). This response is a nonspecific reaction that is triggered by many different stressors both internal and external. When a stressor is perceived by the brain, corticotrophin releasing hormone (CRH) is released from the hypothalamus of the brain. The CRH causes the pituitary gland to release adrenocorticotropic hormone (ACTH) which in turn stimulates the adrenal gland to release glucocorticoids. Glucocorticoids are steroid hormones including cortisol and corticosterone (CORT) (Sapolsky, 2002). Stressors also trigger release of the hormone epinephrine and activation of the sympathetic nervous system. Epinephrine and the sympathetic nervous system activates the flight or fight response that coordinates an immediate response to the stressor (Sapolsky, 2002). Activation of the CRH-ACTH-glucocorticoid axis represents a more long-term response to a stressor.

A short term stress response could be beneficial by enabling the animal to survive a stressful event. For example, glucocorticoids promote physiological and behavioral changes including rapid glucose production that enable an animals to better survive a short-term stressful event (Romero, 2002). However, chronically elevated glucocorticoids could be damaging by causing neuronal cell death and complete reproductive failure (Romero, 2002). Glucocorticoids in high levels could also cause

negative effects on reproductive physiology by inhibiting testicular testosterone synthesis (Denari and Ceballos, 2006) via changes in hormones involved in the hypothalamic-pituitary-gonadal axis such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), and gonadal steroids (Gore, et al., 2006). In addition, the secretion of insulin important for glucose storage is inhibited (Sapolsky, 2002). Also, CORT suppresses wound healing when the animal is in energetically challenged states such as reproduction or resource restrictions, although during times of high resource availability, wound healing is not compromised (French, et al., 2007).

4. Stress Physiology in Amphibians

In most vertebrates basal levels of plasma glucocorticoids change seasonally (Romero, 2002) and are typically higher during the breeding season. Basal patterns of CORT in the crested newt (*Triturus carnifex*) were studied by Zerani and Gobbetti (1993). They found two peaks throughout the year: one in winter at the beginning of the reproduction season, and one in summer, when the newts leave the pond. In the American toad (*Bufo americanus*), edible frog (*Rana esculenta*) and Japanese common toad (*Bufo japonicus*) the highest plasma CORT concentrations corresponded to the time of year when breeding activity occurred (Zerani and Gobbetti, 1993). This pattern also occurred in the Argentine toad (*Bufo arenarum*) (Denari and Ceballos, 2006). In contrast, CORT concentrations did not vary seasonally in the spotted salamander (*Ambystoma maculatum*) (Homan, et al., 2003). Basal levels of CORT also change on a daily basis in most vertebrates, with peaks of CORT preceding the activity period (Zerani and Gobbetti, 1993). Basal levels of glucocorticoids regulate basic metabolic processes.

In addition, CORT increases in response to stressors, and the stress response may change depending on the season (Homan, et al., 2003). For example, after thirty minutes of handling and restraint *A. maculatum* doubled its CORT concentrations during its spring migration to ponds for breeding. However, the CORT concentrations did not change after handling and restraint during the fall as animals migrated to over-wintering sites (Homan, et al., 2003).

Levels of CORT also increase with expression of reproductive behaviors. For example, plasma CORT levels and androgen levels were elevated in male southern toads (*Bufo terrestris*), male North American bullfrogs (*Rana catesbeiana*), male Couch's spadefoot toads (*Scaphiopus couchi*), male green tree frogs (*Hyla cinerea*), and male striped chorus frogs (*Pseudacris triseriata*) engaging in amplexus or calling behaviors (Moore and Jessop, 2003). The increased CORT is thought to help sustain physiologically demanding behaviors (Landys et al., 2006).

Only a few studies have experimentally manipulated CORT levels in amphibians. Injections of CORT into the rough-skinned newt (*Taricha granulosa*) caused rapid suppression of reproductive behaviors. This reproductive behavior suppression occurs within a time frame of a few minutes following CORT injection (Moore, et al., 2005).

CORT added to the aquarium water of pro-metamorphic western spadefoot toad tadpoles (*Spea hammondii*) stimulated foraging behavior, suggesting it is an important regulator of energy balance and food intake (Crespi and Denver, 2004). Exposing CORT to pre-metamorphic *R. pipiens* tadpoles, however, did not alter behavior (Glennemeier and Denver, 2002). The differences between the species could be due to specific metabolic demands due to the ecological conditions unique to each species.

5. Effects of Environmental Degradation on Amphibian Stress Physiology

Coal ash is an environmental stressor in some amphibians. Coal ash contains high concentrations of various heavy metal trace elements such as cadmium, arsenic, selenium, chromium, copper, and barium. In 2000, it was estimated that 120 million tons of coal ash waste was produced annually in the United States (Hopkins, et al., 1997). Coal ash altered *B. terrestris* tadpoles' metabolic rates, grazing success, and ability to avoid predators. Compared to an unpolluted reference site, the CORT levels in the polluted site were significantly elevated. Tadpoles also had higher levels of trace elements in their tissues (Hopkins, et al., 1997). In adult *B. terrestris*, calling behavior is accompanied by an increase in testosterone and CORT. However, in the polluted site subjects, there was no change in CORT or testosterone levels in animals exhibiting calling behavior. Instead, the levels were elevated regardless of the behavior and the calling behavior occurred for a month longer than at the unpolluted site. This change in mating behavior could have negative timing and energetic consequences. Therefore, continuous exposure to contaminants might affect a population in a lethal way (Hopkins, et al., 1997).

6. The Study Organism: *Desmognathus ochrophaeus*

The plethodontid salamander, *D. ochrophaeus*, is a nocturnal semi-aquatic salamander local to western Pennsylvania, specifically concentrated in the Appalachian Mountains and its associated foothills. It ranges from western Virginia and eastern Kentucky in the south to New York in the north. *D. ochrophaeus* lives in moist habitats along streams, springs and seeps. Mating occurs in the spring and fall while

spermatogenesis occurs in the summer months (Benner and Woodley, 2007). Even though there is a high density of salamanders in a streamside habitat, especially compared to other species, they are a largely ignored species of study (Petranka and Murray, 2001). Rocco and Brooks (2000) surveyed 14 watersheds for water chemistry, stream habitat, and salamander assemblages. The 14 watersheds they examined included sites that were chronically acidified by acid mine drainage, episodically acidified, fragmented with high alkalinity, or acid-neutral reference sites. The abundance of salamanders was reduced in acidified sites, although D*. ochrophaeus* were notable in being present in relatively high numbers in acidified streams.

7. Objectives and Hypotheses

The focus of this research is to more fully understand the effects of environmental degradation on the physiological stress response of stream-side salamanders by (1) studying their CORT stress response in the field in an episodically acidified site and an acid neutral site, and (2) studying their behavioral responses to chronically elevated CORT in the laboratory.

Experiment 1: To determine whether acidification affects the physiological stress response, I measured changes in plasma corticosterone after capture and handling in the field in *D. ochrophaeus* from two sites that differed in acidification. Both sites were located in the central Appalachian ecoregion and were approximately three miles apart, with similar climate and habitats. The first site (Linn Run) experiences episodic acidification of pH 4.66 to 5.67 from natural occurring poor buffering capacity. The second site (Camp Run) is acid neutral with a pH of 6.59 to 7.64 (Rocco and Brooks, 2000).

I hypothesized that at the acidified site I would find no stress response. I reasoned that animals were already experiencing a stressful environment from living in an acidified environment which could blunt any additional stress response. On the other hand, I expected to find a stress response in an acid neutral site because the animals were living in pristine conditions and had no reason to blunt their stress response.

Experiment 2: To determine the effects of CORT on behaviors important to survival and reproductive success, I experimentally altered levels of plasma CORT with implants. To do so, male *D. ochrophaeus* were given either an implant containing CORT to chronically elevate CORT, a blank implant with no additional hormone, or an intact control group that did not receive any surgery or implant. Mating, feeding, activity levels in the presence or absence of chemosensory cues were then measured. I hypothesized that animals with chronically elevated CORT would have suppressed expression of behaviors except those most necessary for immediate survival such as the ability to avoid a predator. I predicted that males that received a CORT implant would experience decreased mating and feeding, and increased activity compared to males that received blank implants and intact males.

Methods – Experiment 1

1. CORT Response to Handling in the Laboratory

1.1 Subjects

All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. Collecting permits were obtained from Pennsylvania Fish and Boat Commission for *D. ochrophaeus*. The red-legged salamander (*Plethodon shermani*) was collected from Wayah Bald, Macon County, North Carolina with the assistance of Lynne Houck using collecting permits obtained from North Carolina Department of Wildlife. *D. ochrophaeus* were collected from Linn Run, Forbes State Forest, Westmoreland County, Pennsylvania. Adult male and female *D. ochrophaeus* and adult female *P. shermani* were housed for 7 months (*D. ochrophaeus*) or 5 months (*P. shermani*) in the laboratory with a 14L:10D light cycle. The experiment was conducted in December 2006 from 12:00 hr to 16:00 hr.

1.2 Treatments

To obtain baseline levels of CORT, animals were decapitated and trunk blood was collected with a heparinized capillary tube within 2 minutes of handling. To obtain stress induced levels of CORT in *D. ochrophaeus*, I induced autotomy of the tail tip (a natural anti-predator behavior) by gently pinching the last 10 mm of the tail with forceps. Thirty minutes later, subjects were decapitated and trunk blood was collected within 2 minutes

of decapitation with a heparinized capillary tube. To obtain stress induced levels of CORT in *P. shermani*, the animal, while still in its home box, was placed on a rotator for 30 minutes at 144 RPM. After 30 minutes on the rotator, animals were decapitated and trunk blood was collected with a heparinized capillary tube within 2 minutes of decapitation.

2. CORT Response to Capture and Handling in the Field

2.1 Subjects

D. ochrophaeus were collected by hand from Linn Run in Forbes State Forest, Westmoreland County, Pennsylvania from 20 May to 30 May 2007 (breeding season), and from 22 August to 24 August 2007 (non-breeding season). To minimize potential variation due to time of day, all animals were captured from 14:00 to 17:00 hr. *D. ochrophaeus* were also collected by hand from Camp Run in Forbes State Forest, Westmoreland County, Pennsylvania on 24 August 2007 for an additional non-breeding season sample. Linn Run is episodically acidified (pH 4.66 – 5.67) while Camp Run is acid neutral (pH 6.59 – 7.64) (Rocco and Brooks, 2000). Adult males were classified by the presence of elongated premaxillary teeth and adult females were classified by the presence of vitellogenic eggs visible through the body wall.

2.2 Treatments

To obtain baseline levels of CORT, animals were captured by turning over logs and rocks and seizing by hand. Within 2 minutes of capture, animals were decapitated and trunk blood was collected with a heparinized capillary tube. To obtain stress induced

levels of CORT, animals were captured and placed in a Ziploc bag for either 15, 30 or 60 minutes and then decapitated and blood was collected.

3. Hormone Assays

Field collection took place over a 3 hour period, with 1 hour of transport time. Therefore, blood samples were centrifuged by 4 hours of collection and the plasma fraction was frozen at 20°C in heparinized vials until hormone assays were performed. Hormone levels were measured via radioimmunoassy (RIA) by Dr. Francis Pau from the Endocrine Services Laboratory, Oregon National Primate Research Center.

4. Statistical Analysis

Statistical analyses were performed using SPSS statistical software. The hormone data were analyzed with an ANOVA followed by Student-Newman-Keuls pairwise comparison tests. To satisfy assumptions of parametric tests, the hormonal data were log-transformed before analysis if necessary.

Methods – Experiment 2

1. Subjects

Animals were collected from Linn Run in Forbes State Forest, Westmoreland County, Pennsylvania with permits obtained from Pennsylvania Fish and Boat Commission. All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. For experiment A, male and female *D. ochrophaeus* were collected by hand from 3 May to 9 June 2007. For experiment B, males were collected on 9 September 2007. Adult males were classified by the presence of elongated premaxillary teeth and adult females were classified by the presence of vitellogenic eggs visible through the body wall. At the time of collection, animals were weighed and snout-vent length (length from rostral tip of the snout to the anterior margin of the cloacal vent) was measured. In the laboratory, animals were housed individually in 15 cm x 15 cm Gladware boxes lined with paper towels moistened with ddH_2O of a pH 5.0. Additional moistened paper towels served as cover items. Animals were kept on a 14 hr:10 hr light to dark cycle at 16°C and fed wax worms approximately every 2 weeks. Animals were mated before the start of the experiment to ensure that all animals were reproductively active.

Three treatment groups were used. One group of animals received surgery to insert CORT implants into the body cavity. A second treatment group of animals received surgery to insert blank implants into the body cavity that did not contain any

hormone. To control for surgery and implants, a third group of animals was not subjected to surgery or treatment with an implant (control).

I chose to use a gelling implant developed by French et al. (2007). These implants have been successfully used to elevate plasma levels of CORT in the tree lizard. According to French et al. (2007), the implants deliver hormone in a consistent manner for at least 2 weeks.

2. Preparation of Implants

The implants were prepared by weighing the components in sterile syringes. 1.031 g Poly(ethylene glycol)diacrylate (PEGDA) was weighed out in a sterile syringe. In a second syringe, 0.360 g Pentaerythritol tetrakis 3-mercaptopropionate (QT) was weighed. In a third syringe, 0.464 g of phosphate buffered saline (PBS) pH 7.0 was weighed. For the CORT implants, 60 mg CORT and the PEGDA were added to a microcentrifuge vial and vortexed until the CORT was fully suspended in the PEGDA. The solution was then transferred back to the PEGDA syringe (French, et al., 2007).

The PEGDA and QT were mixed together with 15 transfers between 2 luer loc syringes with the final mixture ending in the QT syringe. This organic solution was mixed with PBS for 30 seconds between 2 luer loc syringes then quickly injected into non sterilized polyethylene tubing diameter 1.67mm. The solution was allowed to dry and harden in the tube overnight. The next morning, the tube was cut away and the implants were cut into 5 mm long cylinders and rinsed in ethanol (French, et al., 2007).

For the blank implants, the above procedure was followed without adding CORT to PEGDA. Implants were used in the surgeries the day they were removed from the

tubing. Pilot data indicated that at 3 weeks, these implants delivered high physiological levels of CORT.

3. Surgeries

Males were anesthetized via immersion in 0.5% MS222 pH 7.0. Surgical tools were sterilized with a FST brand hot bead sterilizer. A small incision, approximately 2 mm long, was made in the lateral wall of the lower abdomen. An implant was inserted into the body cavity and the incision was closed with 2 stitches by 7-0 nylon monofilament. Animals were checked daily for 2 weeks to monitor recovery.

At the time of surgery, all males, including control males, were weighed. Weighing was done after the implant was added to accurately monitor body weight. At the end of the experiment the males were weighed to determine body weight change over the course of the experiment.

Surgeries were conducted in a staggered fashion in order to facilitate behavioral testing. The 30 males were divided into 6 batches of 5 males each (groups A through F). Surgery on each batch was separated by 2 days. The staggered timing of surgeries ensured that all animals were tested behaviorally exactly 2 weeks after surgery, and sacrificed exactly 3 weeks after surgery.

To determine a profile of hormone release, the surgical procedure described above was repeated with an additional group of males. No behavioral experiments were done on this group of animals. Half were sacrificed by decapitation at 1 week and the remaining males were sacrificed at 2 weeks.

4. Courtship and Mating Behavior

In *D. ochrophaeus*, courtship and mating behavior consists of a series of stereotyped behaviors that can last several hours (Arnold, 1976). Sperm transfer is external via a spermatophore. Mating begins when the male orients himself next to the female and attempts to deliver courtship pheromones to the female. If both the male and female are receptive to mating, eventually an advanced stage of courtship is reached called tail straddling walk. In tail straddling walk the female rests her chin on the base of the male's tail with her legs straddling his tail. The pair walks forward in tandem in this position. The male stops and deposits a spermatophore. The pair moves forward until the female's cloacal vent is over the spermatophore. The pair stops, and insemination occurs when the female takes the sperm into her cloacal vent.

To quantify courtship and mating behavior, an experimental male was placed with a reproductive female in a testing chamber (plastic Petri dish, diameter 14 cm) lined with dampened filter paper overnight under dim light at room temperature. Lamps were placed in position to provide enough ambient light for the camera to function while not disturbing the animals. The edge of the testing chamber was covered with a ring of paper so the animal could not see out of the sides of the chamber. Behavior was recorded with time lapse video by recording for 2 seconds every 30 seconds for 10 hours. Recordings began at approximately 20:00 hr and lasted until 6:00 hr. The next morning the presence of a spermatophore and sperm in the female's cloacal vent was assessed visually. Later, videotapes were watched and onset of tail straddling walk was noted. Also, length of tail straddling walk and the number of times the male touched his snout to the female's body was noted. Males were distinguished from females on the videotape by size and color.

Each male was given 3 opportunities to mate with a female. Each male was paired with 3 different females, with 2 nights between pairings. Females were randomized over the treatment groups, and no female was paired more than once with any male.

5. Feeding Behavior

Feeding propensity was measured by providing animals with fruit flies (*Drosophila melanogaster).* Before the start of the experiment, animals were fed 10 fruit flies in the same manner as described below so they gained experience with the feeding task.

Tests were performed at 16°C in each animal's home box. The paper towel used as a cover item was removed from each animal's home box. Ten previously frozen *D. melanogaster* flies were placed on white filter paper (6 cm x 7 cm wedges) and placed in each male's home box at 20:00 hr. The next morning (8:00 hr) the number of *D. melanogaster* left in the box was counted. Tests were repeated twice with 2 days between trials. The number of flies consumed was calculated by subtracting the total number of flies remaining after each of the 2 trials from 20.

6. Locomotor Activity in the Presence of Chemosensory Cues from a Predatory Salamander

First, I developed a behavioral assay using intact female *D. ochrophaeus* to measure activity and predator avoidance. To do so, locomotory activity was measured with a repeated measures design in the presence of a substrate moistened with 1) water, 2) chemosensory cues from predatory salamanders, or 3) chemosensory cues from non-

predatory salamanders. Next I used the behavioral assay to test the effects of surgery and administration of a CORT implant on locomotory activity in the presence of chemosensory cues from a predatory salamander.

Tests were performed under dim light at room temperature from approximately 18:00 to 19:00 hr. Each animal was tested once in a testing chamber (plastic Petri dish, diameter 14 cm) lined with filter paper moistened with chemosensory cues (5 ml of salamander rinse) from a predatory salamander, the spring salamander (*Gyrinophilus porphyriticus*), another night in a chamber containing chemosensory cues from a non-predatory salamander, *P. shermani,* and another night in a chamber moistened with water only. On each night of testing, some animals were exposed to *G. porphyriticus* chemosensory cues, some animals were exposed to *P. shermani* chemosensory cues, and some animals were exposed to the water control. The observer was blind to the treatment.

Before testing, animals were habituated to the testing chambers. First, they were placed into an upside-down testing chamber with the salamander resting on the lid. After an hour, the bottom of each testing chamber was removed, leaving the salamander on the lid, and the bottom was replaced with a new bottom lined with paper moistened with predatory salamander rinse, non-predatory salamander rinse, or water. The testing chamber was gently turned right-side up so the animals rested on the filter paper. This mode of preparing the testing chamber reduced the expression of escape behavior by the subjects because they were habituated to the chambers for an hour before testing began.

Five subjects at a time were videotaped by recording for 2 seconds every 30 seconds for 1 hour. The edges of the testing chambers were covered with a ring of paper

so the animals were visually isolated from one another. Later, videotapes were observed and the location of each animal's head in each of 4 quadrants every 30 seconds was noted. Each animal was tested on each of the chemosensory cues separated by an interval of 2 days.

To prepare the chemosensory cues, *G. porphyriticus* (collected June 2007 Forbes State Forest, Westmoreland County, Pennsylvania), and *P. shermani* (collected August 2006 Wayah Bald, Macon County, NC) were placed in Gladware boxes (15 cm x 15 cm) in 60 mL of ddH_2O for 12 hours at 16°C. At the same time, a Gladware box was prepared with 60 mL of ddH_2O but with no salamander. The presence of a salamander in the ddH_2O lowered the pH from 5.0 to 4.5. Solutions were used within 1 day to minimize degradation. This provided enough solution to run 5 tests.

7. Blood Sampling and Hormone Assays

Blood samples were collected either 3 weeks (Experiment A) or 1 and 2 weeks after surgery (Experiment B) to obtain hormone concentrations. Animals were killed via decapitation at approximately 15:00 hr, and trunk blood was collected in heparinized capillary tubes within 2 minutes of decapitation. The blood was centrifuged and the plasma fraction was frozen at 20°C in heparinized vials until hormone assays were performed. For Experiment A, hormone levels were measured via RIA by Dr. Ignacio Moore from Virginia Polytechnic Institute. Dr. Moore measured testosterone levels in the samples but his hormone assay was not sensitive enough to detect plasma CORT levels. Thus, we did not obtain CORT levels from the animals in Experiment A. The coefficient of variation (CV) for the testosterone RIA was 12.0%.

For Experiment B, CORT levels were measured via RIA by Dr. Francis Pau from the Endocrine Services Laboratory, Oregon National Primate Research Center. Testosterone levels were not measured in samples from Experiment B. The CV for the corticosterone RIA was 1.1%.

8. Mental Gland Size

To examine mental gland size, the heads of the animals were collected during decapitation and processed for cryosectioning. First, the lower jaws were fixed in Bouins overnight. Bouins fixative was chosen because of pilot studies completed that discovered Bouins caused less tearing of the samples during cryosectioning than other types of fixative. After 24 hours, the lower jaws were rinsed twice with ddH_2O and placed in 2 mL DeCal for 2 days. They were then rinsed once with ddH_2O and cryoprotected in 2 mL 30% sucrose (30 g sucrose in PBS) overnight. At this point, 1 mL OCT was added to the vial and allowed to sit on the rotator overnight. Finally, the lower jaws were embedded in OCT, wrapped in foil and frozen at -20°C until cryosectioning.

The lower jaws were cryosectioned at 20 μm thin cross sections and placed on microscope slides for viewing under a microscope. Image-Pro Plus 5.0 software was used to measure the area of each mental gland cross section.

9. Statistical Analysis

Statistical analyses were performed using SPSS statistical software. To analyze mating behaviors a Kruskal-Wallis test was used, with Mann-Whitney pairwise comparisons following. Feeding propensity was measured with a one-way ANOVA followed by Student-Newman-Keuls pairwise comparison tests. Locomotor behavior

was analyzed with a one-way repeated measures ANOVA followed by within subjects contrasts tests. To satisfy assumptions of parametric tests, the hormonal data were log-transformed and then analyzed with a one-way ANOVA followed by Student-Newman-Keuls pairwise comparison tests.

Results – Experiment 1

1. CORT Response to Handling in the Laboratory

CORT was elevated in both male and female *D. ochrophaeus* induced to autotomize their tail tips compared to controls ($F_{1, 8} = 5.574$, $P = 0.046$). Females had lower overall CORT levels compared to males ($F_{1, 8} = 11.046$, $P = 0.010$) (Figure 1). There was no significant change in CORT levels in *P. shermani* placed on a rotator for 30 minutes compared to control animals ($F_{2, 7} = 0.369$, $P = 0.704$) (Figure 2).

2. CORT Response to Capture and Handling in the Field

In animals collected from Linn Run during the breeding season, capture and handling did not elicit a change in CORT levels ($F_{2, 42} = 1.384$, $P = 0.262$) (Figure 3). Females had lower overall CORT levels compared to males ($F_{1, 42} = 39.627$, $P < 0.001$) (Figure 3). In animals collected from Linn Run during the non-breeding season, capture and handling did not elicit a change in CORT levels ($F_{2, 27} = 1.653$, $P = 0.210$) (Figure 4).

CORT levels in animals collected from Camp Run differed among the 3 groups ($F_{2, 12} = 4.432$, $P = 0.036$) (Figure 4). There was a significant CORT increase 30 minutes after capture and handling ($P < 0.05$, Student-Newman-Keuls test). By 60 minutes after capture and handling, CORT levels had returned to baseline levels (Figure 4).

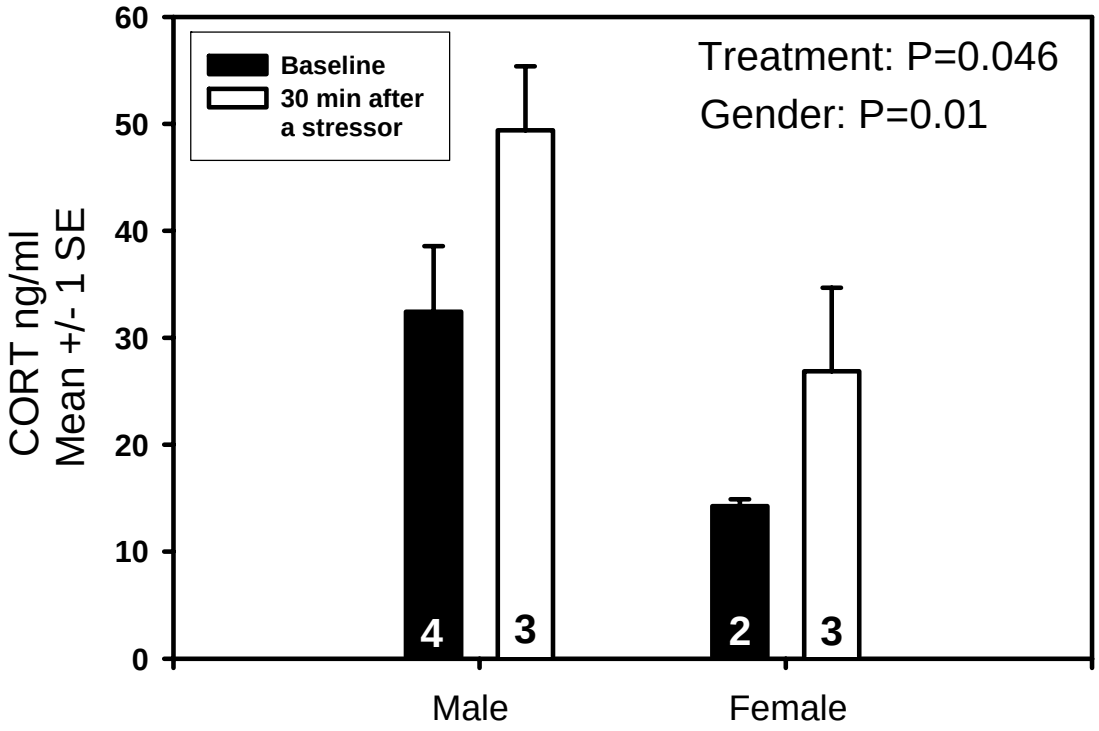

Figure 1. Plasma CORT levels in laboratory housed male and female *D. ochrophaeus*. Subjects were either not exposed to tail autonomy or exposed to tail autonomy, and blood was collected 30 minutes afterwards. Significant main effects are reported on top of graph. Sample sizes are indicated in bars.

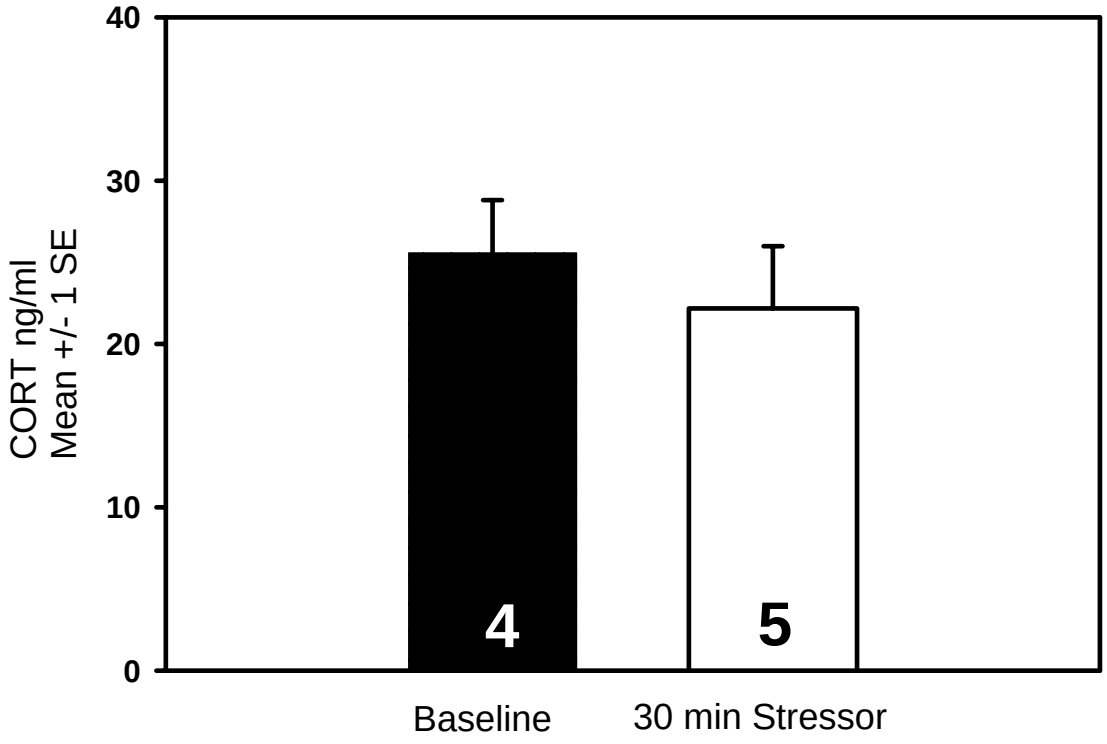

Figure 2. Plasma CORT levels in laboratory housed female *P. shermani*. Subjects were bled immediately (baseline), or exposed to 30 minutes on a rotator and then bled (30 min Stressor). Sample sizes are indicated in bars.

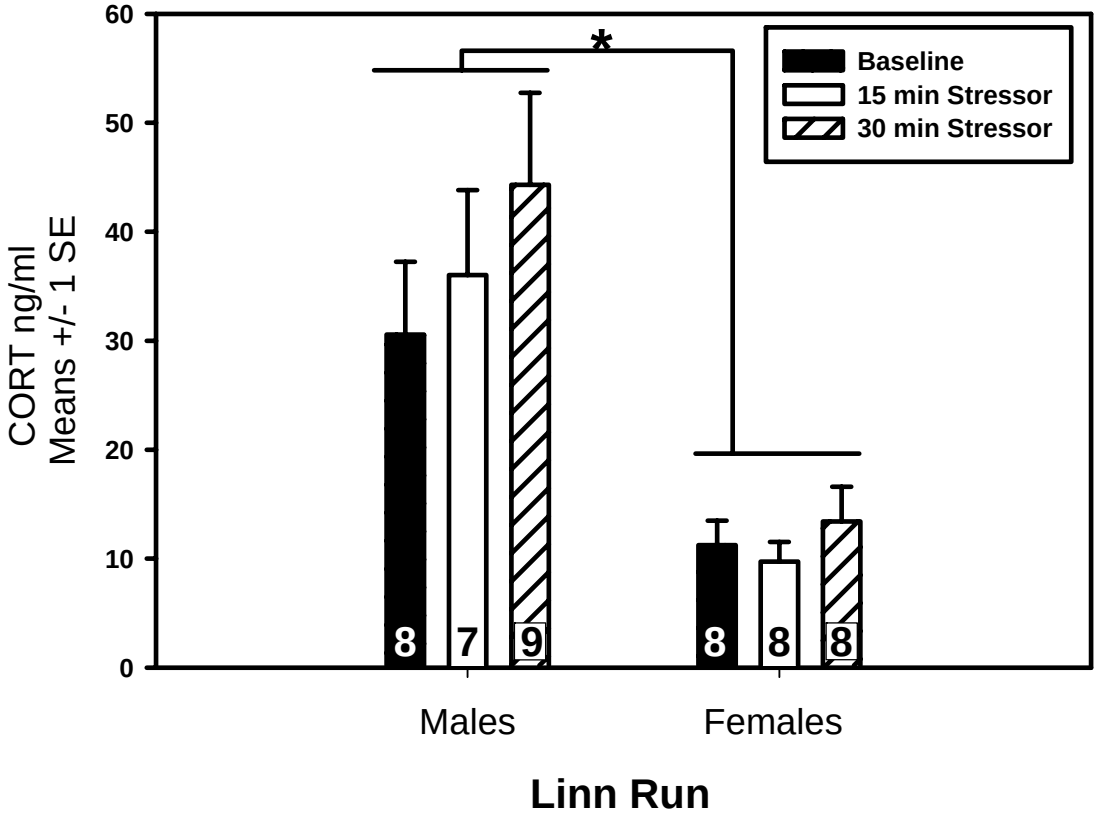

Figure 3. Plasma CORT levels in male and female *D. ochrophaeus* collected from the field during the breeding season. Subjects were collected from Linn Run, an episodically acidified stream. Subjects were bled immediately, 15 minutes or 30 minutes after capture. There was no effect of time from capture to bleed on plasma CORT, although females had lower CORT than males (*$P < 0.001$). Sample sizes are indicated in bars.

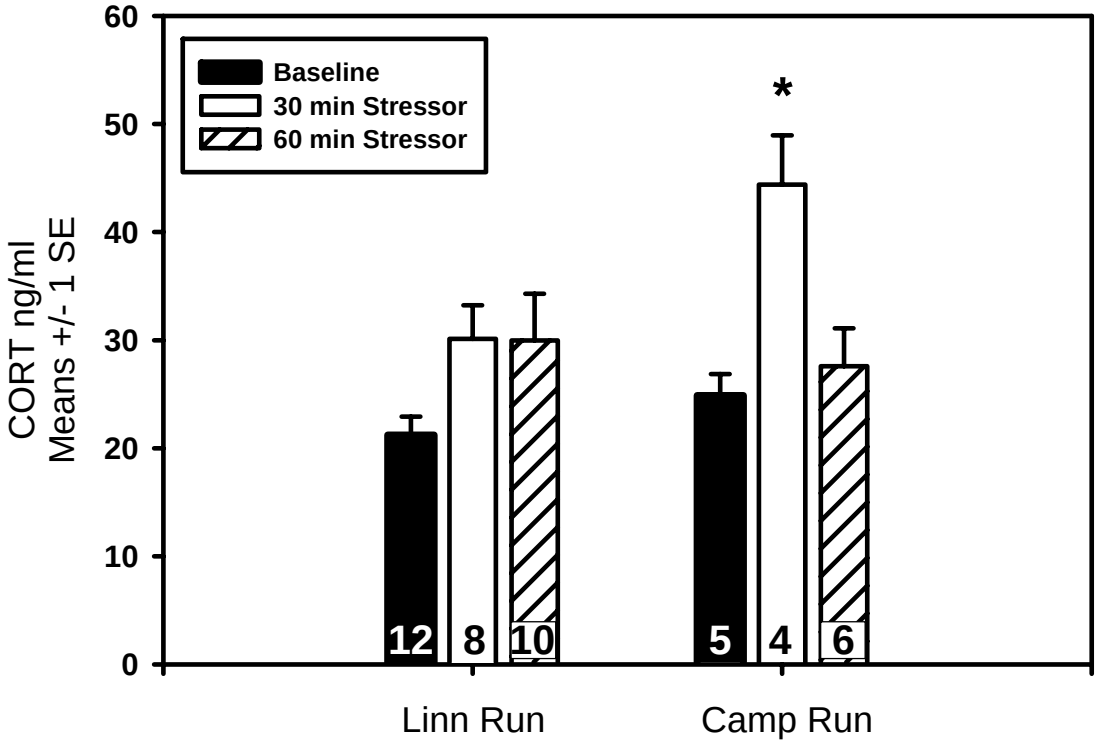

Figure 4. Plasma CORT levels in males collected from the field during the non-breeding season. Subjects were collected from either Linn Run (episodically acidified) or Camp Run (acid neutral). Subjects were bled immediately, 15 minutes or 30 minutes after capture. In males from Linn Run, there was no effect of time from capture to bleed. In males from Camp Run, plasma CORT was elevated at 30 minutes compared to baseline and 60 minutes after capture (* : $P < 0.05$, Student-Newman-Keuls). Sample sizes are indicated in bars.

Results – Experiment 2

1. Courtship and Mating Behavior

The percent of males that reached tail straddling walk differed among the 3 groups ($\chi^2 = 8.46$, P = 0.015) (Figure 5). There was a trend for reduced tail straddling walk in CORT implanted males relative to blank implanted males (U = 30.0, P = 0.057). There was no difference between blank implanted and intact males (U = 45.0, P = 0.317). There was a trend for CORT implanted males to have reduced levels of spermatophore deposition relative to blank implanted males (U = 30.0, P = 0.057) (Figure 6). The percent of males that inseminated a female differed among the 3 groups ($\chi^2 = 10.73$, P = 0.005) (Figure 7). There was a trend of reduced insemination in CORT implanted animals relative to blank animals (U = 35.0, P = 0.067). There was no difference between blank implanted and intact animals (U = 30.0, P = 0.081).

There was no difference in the number of times each male touched his snout to the female's body before the onset of tail straddling walk among the three treatment groups ($F_{2, 20} = 1.152$, P = 0.336). The mean +/- SEM number of times each male touched his snout to the female's body before the onset of tail straddling walk for CORT implanted animals was 72.5 +/- 11.7 times touched, for blank implanted animals was 61.7 +/- 8.8 times touched, and for intact animals was 122.2 +/- 41.6 times touched. There was also no difference in latency to tail straddling walk in those animals that engaged in tail straddling walk. The mean +/- SEM latency to tail straddling walk of those that engaged in tail straddling walk for CORT implanted animals was 351.58 +/- 83.19 min,

for blank implanted animals was 252.51 +/- 54.66 min, and for intact animals was 315.22 +/- 138.72 min.

2. Feeding Behavior

The number of flies eaten (Figure 8) differed among the 3 groups ($F_{2, 27} = 9.315$, $P = 0.001$). Both CORT and blank implanted animals ate fewer flies than the intact group ($P < 0.05$, Student-Newman-Keuls test).

3. Locomotor Activity in the Presence of Chemosensory Cues from a Predatory Salamander

In intact females exposed to either a substrate moistened with water or a substrate moistened with chemosensory cues from either a predatory or a non-predatory salamander, activity levels depended on the chemosensory stimulus ($F_{2, 18} = 3.646$, $P = 0.047$) (Figure 9). Relative to the water control, activity was reduced in the presence of *G. porphyriticus* chemosensory cues ($F_{1, 9} = 5.654$, $P = 0.041$) but not in the presence of chemosensory cues from *P. shermani* ($F_{1, 9} = 0.07$, $P = 0.80$).

There was no difference in activity level among intact, blank implanted, or CORT implanted males ($F_{2, 27} = 0.004$, $P = 0.996$) (Figure 10). However, activity in the presence of chemosensory cues from *G. porphyriticus* was reduced relative to the water control in all males, regardless of treatment ($F_{1, 27} = 6.406$, $P = 0.018$). There was also no difference among the groups in regards to order of chemosensory cue presentation ($F_{1, 24} = 1.827$, $P = 0.189$).

4. Testosterone Levels

There was a difference in plasma testosterone levels among all five treatment groups ($F_{4, 38} = 5.79$, $P = 0.001$) (Figure 11). Plasma testosterone levels were reduced in all animals that received blank or CORT implants relative to intact animals ($P < 0.05$, Student-Newman-Keuls test).

5. CORT Levels

Treatment groups differed in plasma CORT ($F_{5, 33} = 24.93$, $P < 0.001$) (Figure 12). Plasma CORT was elevated one week after surgery but declined to baseline levels by two weeks after surgery ($P < 0.05$, Student-Newman-Keuls test).

6. Mental Gland Size

There was no difference among the 3 treatment groups in mental gland size ($F_{2,19} = 0.054$, $P = 0.948$). The mean +/- SEM mental gland size for CORT implanted animals was 2.73 +/- 0.53 g, for blank implanted animals was 2.80 +/- 0.57 g, and for intact animals was 2.83 +/- 0.45 g.

6. Body Weights

There was no difference among the 3 treatment groups in initial body weight ($F_{2, 27} = 0.119$, $P = 0.888$) or final body weight ($F_{2, 17} = 0.098$, $P = 0.907$). The starting body weight was 0.953 +/- 0.057 g and the final body weight was 0.761 +/- 0.055 g (mean +/- SEM).

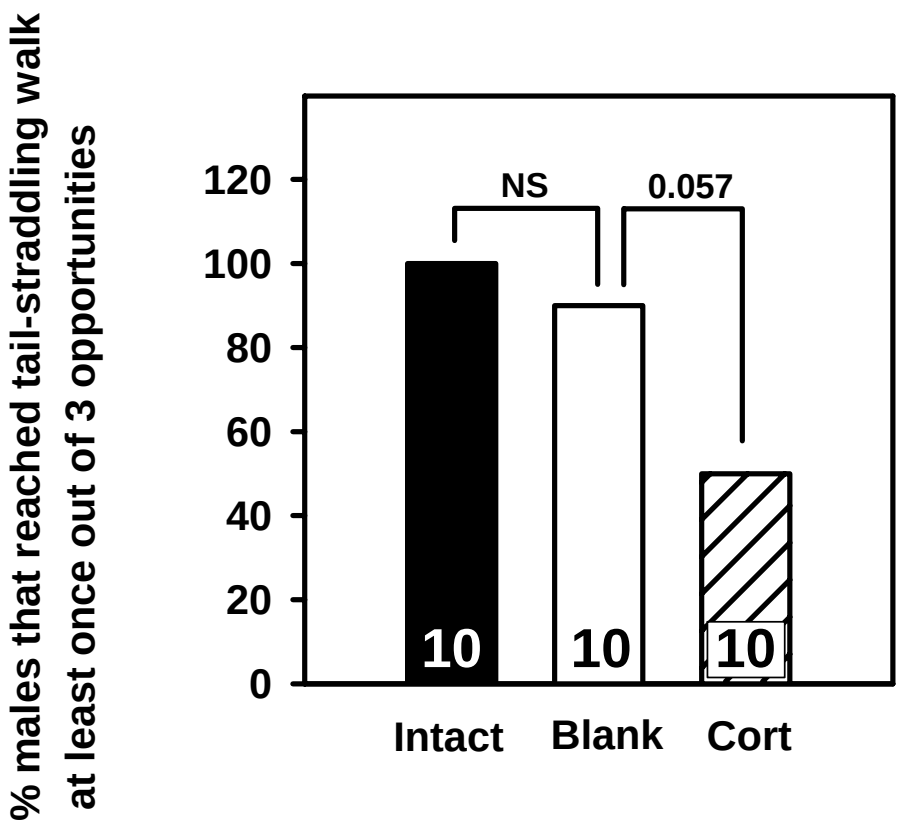

Figure 5. Tail-straddling walk in intact, blank implanted, and CORT implanted males. Sample sizes are indicated in bars. Results of Mann-Whitney pairwise comparison tests are shown.

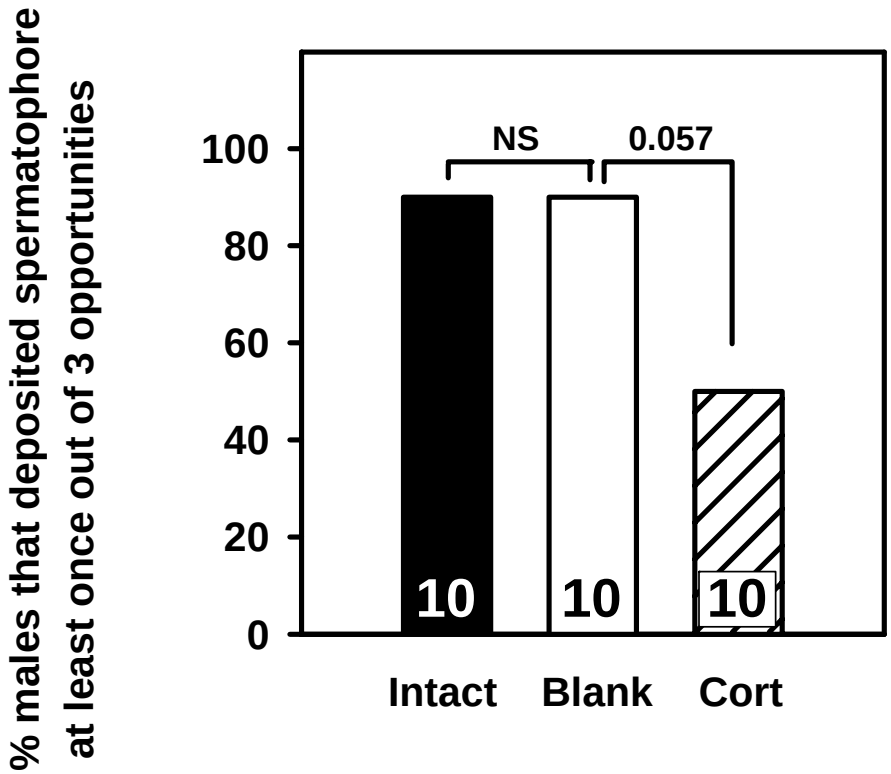

Figure 6. Spermatophore deposition frequency for intact, blank implanted, and CORT implanted males. Sample sizes are indicated in bars. Results of Mann-Whitney pairwise comparison tests are shown.

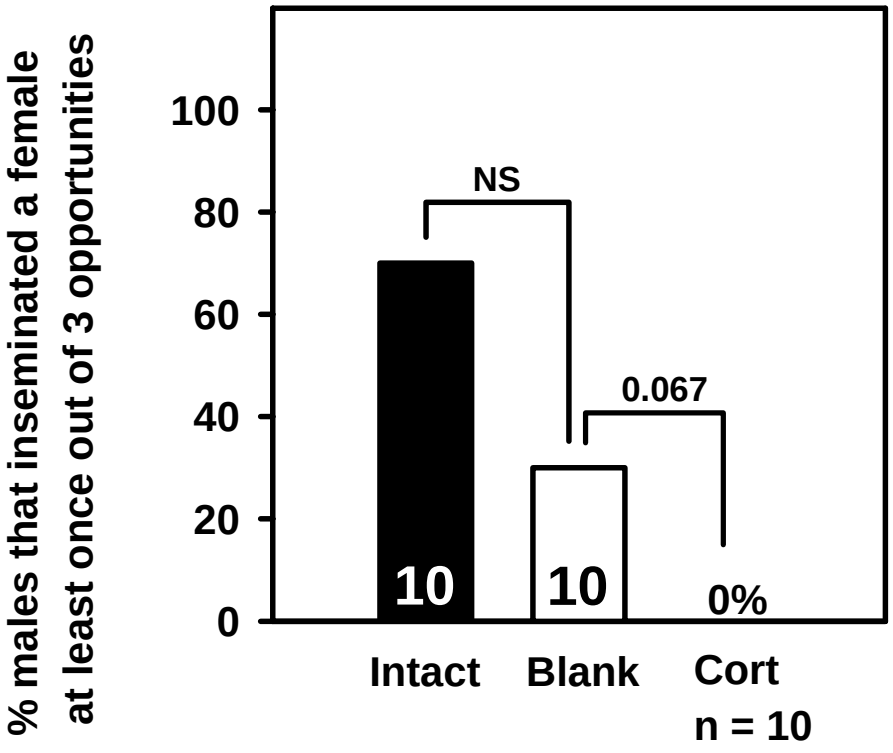

Figure 7. Frequency of inseminations by intact, blank implanted, and CORT implanted males. Sample sizes are indicated in bars. Results of Mann-Whitney pairwise comparison tests are shown.

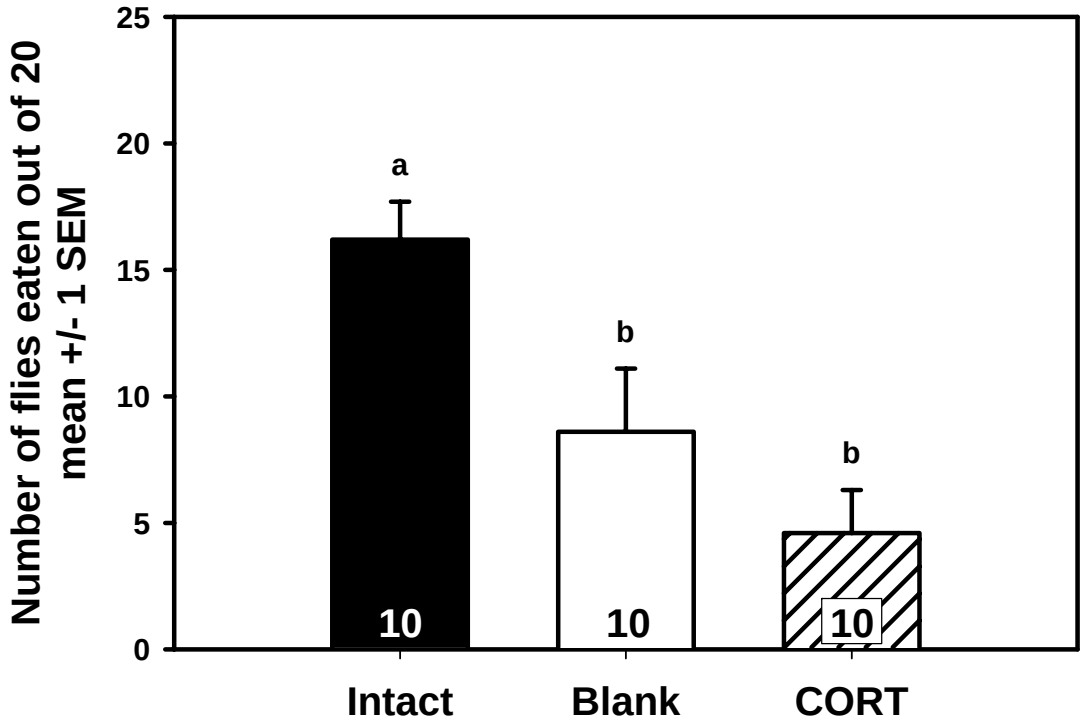

Figure 8. Number of flies eaten out of 20 for intact, blank implanted, and CORT implanted males. Sample sizes are indicated in bars. Different letters denote statistically significant differences, $P < 0.05$, Student-Newman-Keuls test.

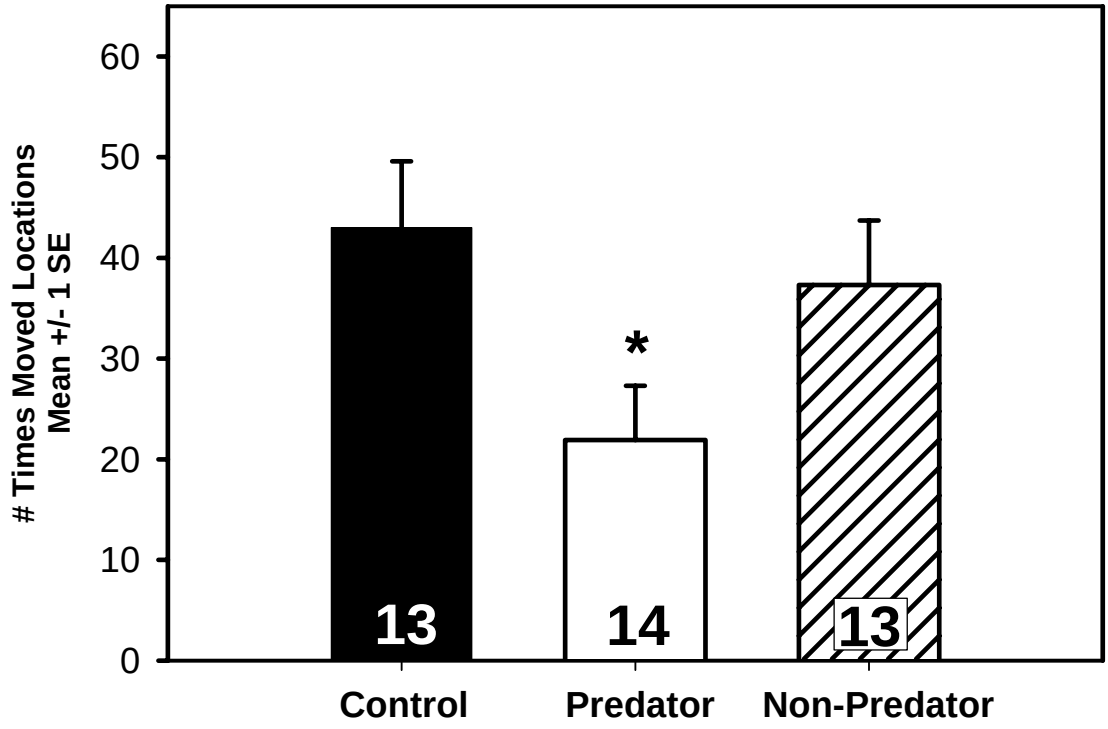

Figure 9. The number of scans each female was found in a different quadrant in the presence of chemosensory cues from a predatory salamander (*G. porphyriticus*) and a non-predatory salamander (*P. shermani*). Sample sizes are indicated in bars. *: P = 0.041 relative to the water control.

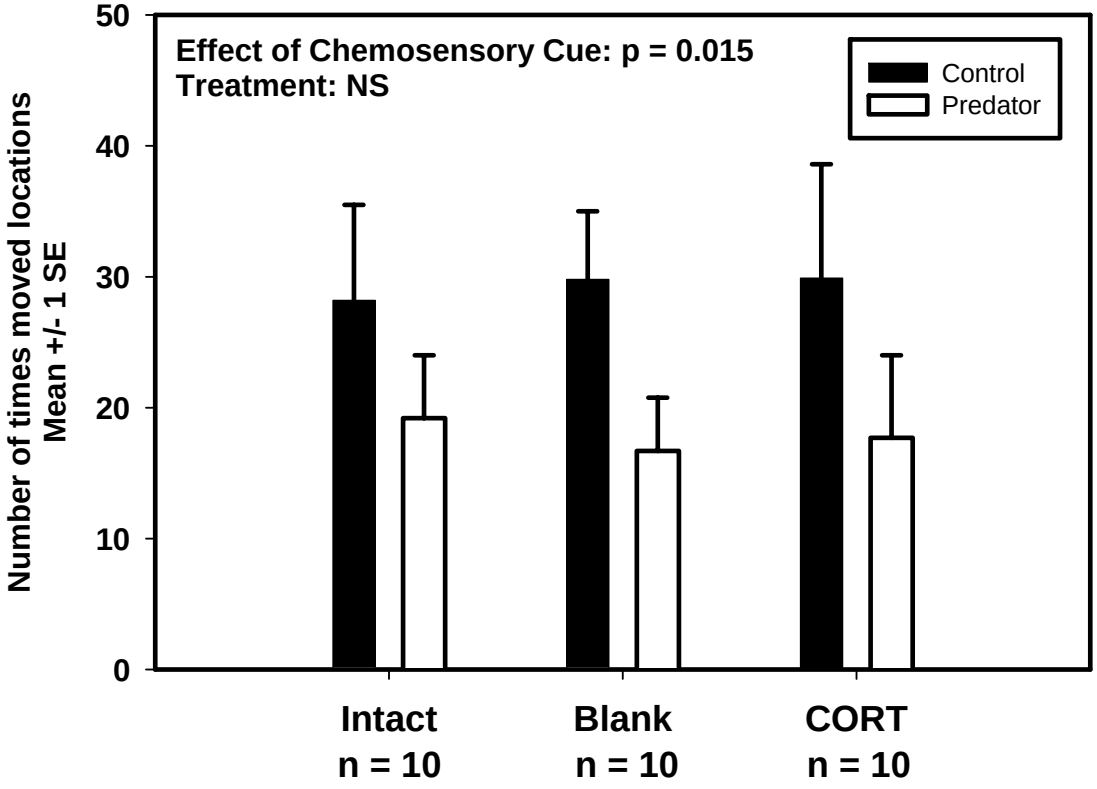

Figure 10. The number of scans each male was found in a different quadrant of the testing chamber on substrates moistened with chemosensory cues from a predatory salamander (*G. porphyriticus*) or water (control). Although there was no significant effect of treatment, all males had reduced activity in the presence of chemosensory cues from a predator. Significant main effects are reported at top of graph.

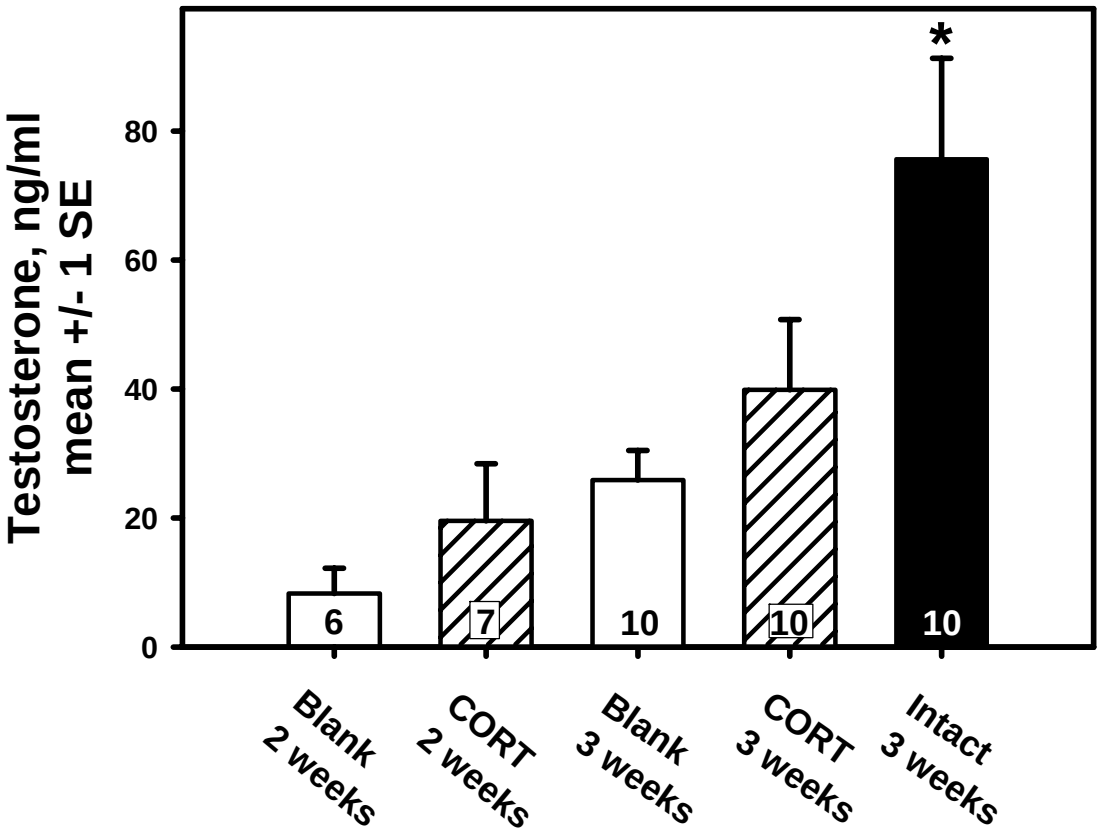

Figure 11. Plasma testosterone levels in intact, blank implanted, and CORT implanted males 2 and 3 weeks after surgery. *: $P < 0.05$ compared to all other groups, Student-Newman-Keuls test.

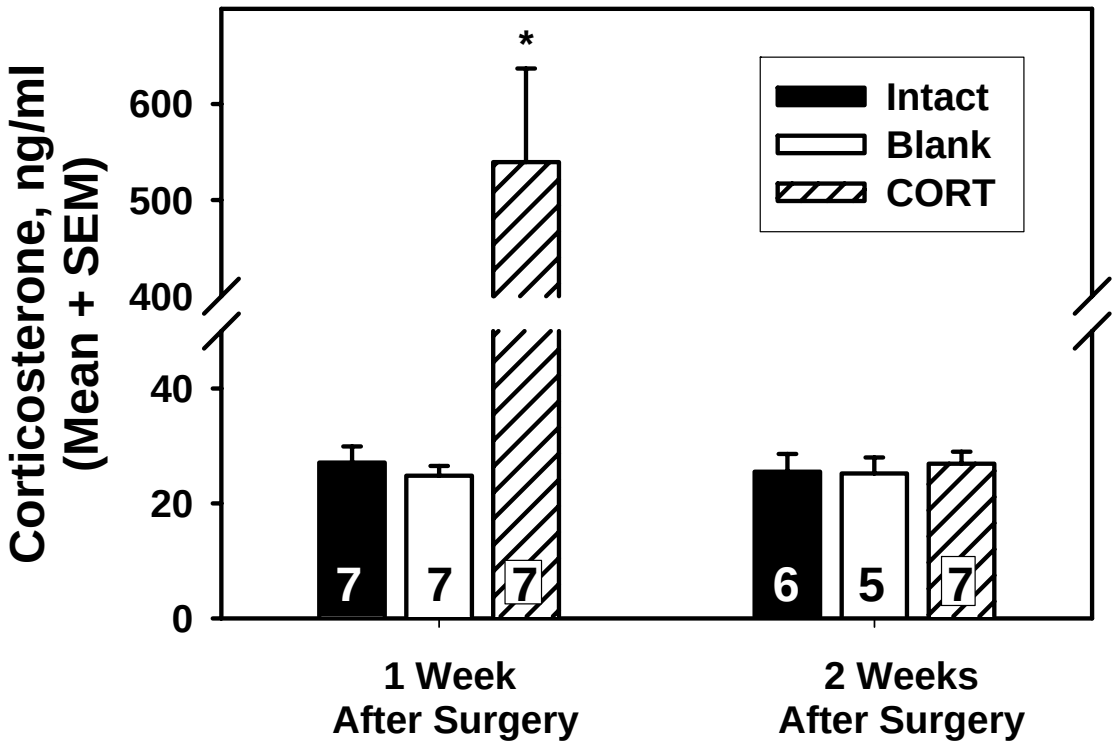

Figure 12. Plasma corticosterone levels in intact, blank implanted, and CORT implanted males 1 week and 2 weeks after surgery. Sample sizes are indicated in bars. *: $P < 0.05$, Student-Newman-Keuls test.

Discussion

Experiments were completed to more fully understand the effects of environmental degradation by acidification on the physiological stress response of stream-side salamanders. They were completed by studying the CORT stress response in the field, and by studying the influence of elevated CORT on behaviors in laboratory-housed animals. I found that animals living in an acidified watershed had a blunted stress response compared to those found in an acid neutral site. Also, there was a trend for an increase in the stress hormone CORT to reduce several aspects of mating behavior and feeding. These results provide evidence that acidification blunts the normal stress response such that animals may not be able to respond to additional stressors. Therefore, it is important to clean watersheds of environmental degradation by acidification due to the potential negative impact of local watershed species. Also, environmental degradation that potentially causes elevation in CORT may adversely impact behaviors important for reproduction. These results will be discussed in more detail below.

1. Experiment 1 – Stress Response in the Field

First, I measured plasma CORT levels in *D. ochrophaeus* after capture and handling to determine whether living in an acidified stream habitat affected their CORT stress response. I measured plasma CORT in animals from two sites that were either episodically acidified (Linn Run) or acid neutral (Camp Run). The study sites were similar in that they were both in the same ecoregion and had similar available habitats,

with the major difference being the dissimilar pH values between the two sites. As expected, for those animals collected from acid-neutral Camp Run, there was a significant CORT increase 30 minutes after capture and handling that returned to baseline levels by 60 minutes. In contrast, capture and handling did not elicit a change in CORT levels in animals collected from Linn Run during both the breeding and non-breeding season for either males or females. The hypothesis was supported that no stress response would be found in animals living in an acidified site. It is possible that chronic stress due to acidification caused the HPA axis to become insensitive to further stressors (Rich and Romero, 2005). Interestingly, autotomizing the tail tips of males in the laboratory that were originally collected from Linn Run does cause a CORT increase 30 minutes after autotomy, suggesting that the blunting of the stress response does not occur if the animals are in the laboratory and in an acid neutral environment; for while the ddH_2O used in their home boxes has a pH of 5.0, in approximately 24 hours, the animals regulated their surroundings to raise the pH to 6.0 to 7.0.

My results are similar to those found in other amphibians. In male spotted salamanders, *A. maculatum*, animals collected in areas of disturbed habitat had lower baseline and stress induced CORT concentrations compared to those from an undisturbed site (Homan, et al., 2003). My results also support the findings by Hopkins, et al. (1997) who reported that in adult southern toads, *B. terrestris*, there was no increase in CORT levels after capture in animals that were exposed to environmental degradation by coal combustion waste, while there was an increase in CORT levels in uncontaminated sites. They also reported how calling behavior in *B. terrestris*, which is a behavior in males to attract females for mating, is affected by polluted sites. The toad's calling behavior was

accompanied by an increase in testosterone and CORT in sites uncontaminated by coal ash waste; however, in polluted sites there was no change in CORT or testosterone levels during calling behavior. Assuming an increase in testosterone and CORT is necessary for proper calling behavior; a blunted hormone response could have negative effects on their behavior and mating. However, my study is the first to examine the effects of acidification, a common feature of many stream habitats in western Pennsylvania, on the CORT stress response.

Future directions for this experiment would examine additional watersheds to determine if the results found in this study are unique to Linn and Camp Run or are found more generally in other watersheds impacted by acidification. To this end, Rocco and Brooks (2000) have identified 14 different watersheds, all within the same ecoregion of western Pennsylvania, representing an acid-alkaline gradient that could be sampled from. The sites have been highly characterized. Another important future direction would be to determine whether acidification per se or a correlated aspect of acidification is responsible for the blunted stress response. In addition to there being a pH difference between Linn and Camp Run, there are also other water chemistry differences. A table modified from Rocco (2007) lists some of these differences.

Table 1. Water chemistry for Muddy, Linn and Camp Runs (Rocco, 2007).

Condition	Stream	Water Chemistry (Min - Max)				
		pH	$CaCO_3$ mg/l	NO_3-N mg/l	Fe mg/l	Al mg/l
(AMD) High acid	Muddy	2.98 4.39	< 0.05	0.005 0.344	0.045 7.0	1.469 62.82
Episodic Low acid	Linn	4.66 5.67	< 0.05 1.60	0.219 0.323	0.039 0.2	0.084 0.377
Reference (non-acidc)	Camp	6.59 7.64	8.96 20.29	0.608 0.799	0.024 0.076	0.04 0.061

Research on the brown trout (*Salmo trutta*) show an example of how heavy metal can affect the stress response. Fish that were severely confined for a short period in water contaminated with Cd and Zn had a blunted stress response compared to those fish confined in uncontaminated water (Norris et al., 1999). These findings show the importance of determining what characteristics of the water are affecting the stress response of the animals.

2. Experiment 2 – Behavioral Responses to Elevated CORT

Next, I manipulated CORT in male *D. ochrophaeus* to determine how CORT affected mating, feeding, and activity behaviors. Male *D. ochrophaeus* received either an implant to chronically elevate plasma CORT, a blank implant with no additional hormone, or an intact control group that did not receive any surgery or implant. I hypothesized that males who received a CORT implant would experience decreased mating and feeding, and increased activity behaviors compared to males who received a blank implant and intact males. I videotaped courtship encounters and examined both the early stages of courtship as well as the final stages of courtship. I found no difference in early courtship behaviors including the number of times each male touched his snout to the female's body before the onset of tail straddling walk or in latency to tail straddling walk in those animals that engaged in tail straddling walk between the 3 treatment groups – indicating that CORT had no effect on the behaviors and/or timing leading to tail straddling walk. However, while not statistically significant, I found a trend of reduced percentage of animals that performed tail straddling walk, spermatophore deposition, and insemination of females in CORT implanted males relative to blank implanted males. These results show that while all aspects of courtship were not affected by chronically

elevated CORT, insemination, the actual determination of successful mating, was eliminated in the CORT implanted animals.

In addition to observing behavior and noting hormone levels, the mental gland was examined using histological methods to see if behavior differences could be attributed to differences in mental gland size. The mental gland produces courtship pheromones that are delivered to the female's olfactory system during tail straddling walk and helps elicit female receptivity to the male. Mental gland size was roughly correlated with plasma testosterone levels, and changed seasonally such that they were largest during the mating season in a related salamander, the red-cheeked salamander (*Plethodon jordani*) (Woodley, 1994). Regardless of treatment or mating success, however, there was no difference in the size of the mental gland among groups. This indicates that differences in behavior were not due to differences in mental gland size and potentially pheromone production.

These data support the findings reviewed by Moore and Miller (1984) who found CORT injections rapidly suppressed male reproductive behaviors (amplectic clasping) in the newt, *T. granulosa* within minutes. However, Moore and Miller (1984) examined rapid effects of CORT acting through a novel glucocorticoid receptor mechanism, while this research examined chronic effects of CORT on mating. Interestingly, there has been no previous study investigating the effects of acute stress or CORT administration on courtship behaviors in any amphibian other than that of *T. granulosa*. My research on *D. ochrophaeus* is therefore very important due to its novel contribution to the field.

The next behavior examined to see how it was affected by increased CORT was feeding behavior. The number of flies eaten was decreased in both CORT and blank

implanted animals compared to the intact group, however, this decrease in feeding did not lead to a decrease in body weight over the course of the experiment.

Finally, I measured activity in the presence or absence of chemosensory cues from the predatory salamander *G. porphyriticus*. There was no difference in activity level among the 3 treatment groups in the presence of the predator *G. porphyriticus* chemosensory cues. Therefore, CORT treatment did not affect activity or predator avoidance. However, in all treatment groups, activity level was decreased in the presence of chemosensory cues from the predator relative to the ddH_2O controls. It should be noted there was a decrease in activity in the presence of a predatory salamander compared to a non-predatory salamander, *P. shermani*. Thus I have developed a useful assay for measuring predator avoidance.

These results correspond to the results found by Dodd (1990) which show that when *D. ochrophaeus* were exposed from their cover in the field, they exhibited immobile behavior. This immobile behavior is thought to be a defensive mechanism to avoid detection because the dusky salamanders lack noxious secretions and structural antipredator defenses.

To verify that CORT implants were elevating CORT levels as expected, plasma CORT was measured in all subjects. Also, since elevated CORT can decrease activity of the hypothalamic-pituitary-gonadal axis to decrease testosterone levels, plasma testosterone was also measured. Plasma testosterone levels were reduced in blank and CORT implanted males 2 weeks and 3 weeks after surgery relative to intact animals. Since the blank implanted animals and the CORT implanted animals both had reduced plasma testosterone, this result could be a residual effect of the surgery itself, or it could

be some effect of the implant. Since testosterone is known to promote male mating behavior, the reduced mating behavior observed could be due to the reduced testosterone levels (Benner and Woodley, 2007). However, there was a reduction in mating behavior in CORT implanted animals relative to the blank implanted animals despite similar testosterone levels. Therefore, it is unlikely that the reduction in mating behavior in CORT implanted males was due to decreased testosterone.

Plasma CORT levels were increased in the CORT implanted animals relative to the other treatment groups by one week after implantation. This confirms that the implants were delivering CORT; however, by two weeks after surgery, the CORT levels were similar to levels in intact males. This implies that the behavior changes could be from previous action of CORT. Also, the CORT levels delivered by the implants were 10 times higher than average levels measured in the field, however, we have measured CORT as high as 264 ng/ml in the field in non-stressed animals (Woodley, unpublished). Therefore, the CORT levels delivered by the implants could potentially be experienced by animals in the field.

It should be noted that I found some effects of the implants themselves. For example, I found a trend for a decrease in insemination of females in the blank implanted animals. I also found that feeding was reduced in blank implanted males. These data suggest that the implants themselves were affecting behaviors. The implant design was adapted from French, et al. (2007) who utilized a novel implant in which CORT was dissolved in a polymer that solidified within a few minutes of mixing. Since their study organism was tree lizards (*Urosaurus ornatus*), they were able inject the polymer subcutaneously which allowed the polymer to solidify under the skin such that animals

did not undergo surgery. Since salamanders lack a subcutaneous space, the delivery of the implants had to be altered. Instead of having the implants inserted under the skin before they were hardened, I allowed the implants to harden outside of the body. They were then inserted into the body cavity via surgery. Because the delivery method and study organism was altered, the blank implant results found in this study could be attributed to those differences. Also, there could be a potential toxic effect due to the toxic gelation inhibitors 4-methoxyphenol (MEHQ) and 2,6-di-tert-butyl-p-cresol (BHT) added to the polymers to prevent gelation during manufacture and shipping. French, et al. (2007) suggested that these toxic effects were minimal in animals larger than 5 g; however, *D. ochrophaeus* are significantly smaller (0.5 – 1.5 g). However, there was no difference in activity, early stages of mating, or in body weight between blank-implanted and intact males, so it is not clear how the implants are affecting behavior.

Another problem with the implants was the dose of CORT that was delivered and the dynamics of release. French, et al., (2007) reported that the gelling implants achieved constant, physiological, sustained concentrations of plasma CORT for 2 weeks post-injection in lizards. I found, however, that levels were very high at one week and dropped rapidly thereafter. Nevertheless, this profile of CORT release could parallel what we would see in the field in animals that experience episodic acidification.

Because of the problems with the gelling implants, a study should be conducted using a different type of hormone implant to see if my results can be replicated. An alternate type of implant is Silastic implants. Studies using Silastic implants show that the implants themselves do not affect behaviors (Benner and Woodley, 2007). By using the Silastic implants, the effects of the blank implants could be eliminated. In addition,

pilot studies would need to be conducted to ensure that the Silastic implants are delivering physiological levels of CORT.

3. Conclusions

In conclusion, environmental degradation by acidification potentially influences the stress axis as found with the blunted CORT response of those animals found in the episodically acidified Linn Run compared to the acid neutral Camp Run. Theoretically, the animals living in Linn Run were already in a state of stress due to the environmental degradation present, which blunted any further CORT increases to prevent any additional stress; similar to the response found in *A. maculatum* (Homan et al., 2003). Those animals found in Camp Run, however, lived in an environment free of acidification and the stress response occurred as normally expected. In addition, chronic elevation of CORT (an increase of CORT for at least 1 week) caused a trend of reduced expression of mating behaviors, specifically tail straddling walk and insemination success. These behaviors are essential for male reproductive success. Ultimately, further research would investigate how environmental degradation (specifically degradation associated with acidification) affects behaviors in free living animals via the effects on stress physiology. If acidification causes the same behavior changes in the wild that were found with the CORT implants, it will support the necessity of cleaning the watersheds that are degraded by acidification, many of which are found in Western Pennsylvania.

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